

ORIGINAL ARTICLE

Controlled Epstein–Barr virus reactivation after allogeneic transplantation is associated with improved survival

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Abstract

Epstein–Barr virus reactivation (EBV-R) frequently occurs in patients having allogeneic hematopoietic stem cell transplantation (HSCT). We evaluated the impact of controlled EBV-R on survival of 190 patients (114M/76F, median age: 51 yr, range 18–69), having HSCT for hematological malignancies (105 acute leukemias and myelodysplasias, 71 lymphoproliferative disorders, 14 others). Overall survival (OS) and progression-free survival (PFS) were compared between patients with and without EBV-R. Of 138, patients had reduced-intensity conditioning regimen. Various stem cell sources (141 PB, 33 umbilical cord blood and 16 bone marrow) were used. Patients with EBV-R had longer PFS and OS than those without EBV-R: PFS at 2 yr 69% vs. 51% and at 5 yr 47% vs. 38% ($P < 0.04$); OS at 2 yr 76% vs. 64% and at 5 yr 63% vs. 47% ($P < 0.001$). The use of rituximab had no impact on OS and PFS, but it reduced the intensity of GVHD, despite the fact that TRM was not significantly different between the two groups of patients. So, rituximab may have an additional effect to other factors on PFS and OS. In multivariate analysis, antithymocyte globulin administration was not a significant factor for PFS ($P = 0.68$) and for OS ($P = 0.81$). Circulating NK cells were significantly increased by 22% ($P = 0.03$) in EBV-R patients with no differences for other parameters. Controlled EBV-R in the setting of HSCT is associated with better OS and PFS, with a significant increase in circulating NK cells.

Key words Epstein–Barr virus; allogeneic stem cell transplantation; NK cells

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B lymphocytes infected by the Epstein–Barr virus (EBV), a γ -herpes transforming virus endemic in humans, are strongly controlled by the immune system, with the persistence of low counts of EBV-infected B lymphocytes in 98% of healthy individuals (1, 2). Under immunosuppressive drugs, EBV-transformed B lymphocytes can expand *in vivo* and cause life-threatening diseases, particularly post-transplantation lymphoproliferative disease (PTLD) (3). The transforming activity of EBV on B cells is due to EBV latent membrane protein 1, which mimics constitutive CD40 activation in B cells (4). Allogeneic hematopoietic stem cell transplantation (HSCT), an effective therapy for patients with hematological malignancies, requires severe immune depletion, particularly in the early phase after HSCT. Several

risk factors for EBV-reactivation (EBV-R) have been described, such as the use of unrelated or mismatched related donors, the occurrence of graft-versus-host disease (GVHD) and its prophylaxis or T-cell depletion, in particular with antithymocyte globulin (ATG) or with anti-CD3 monoclonal antibody (5). Early diagnosis of EBV-R is crucial for successful therapy. Post-transplant monitoring of EBV viral load (VL) in peripheral blood (PB) by polymerase chain reaction (PCR) is effective in predicting patients at risk of EBV-PTLD. Weekly testing of EBV-VL by PCR has shown that EBV-R occurs in up to 50% of patients having HSCT, but the risk of EBV-PTLD is limited to patients with higher EBV-VL (6). The reduction or withdrawal of immune suppression is a well-known first-line therapeutic approach to

control EBV-R, but its use in HSCT patients has been limited by the fear of inducing or worsening acute GVHD and by the speed of EBV replication. The use of rituximab, a monoclonal anti-CD20 antibody, in association with careful follow-up of EBV-VL is effective in reducing EBV-infected B lymphocytes, EBV-VL, and emergence of EBV-PTLD (7). Controlling EBV-R by rituximab was suggested to improve overall survival (OS), but with no definitive demonstration (8). The aim of this study was to compare, in a large homogeneous population of patients having HSCT, progression-free survival (PFS) and OS according to EBV status and other different factors, including administration of rituximab or ATG and circulating lymphocytes.

Material and methods

Patient population and clinical characteristics

Data from all the patients having HSCT in the Hematology Department of the Montpellier University Hospital were prospectively registered. One hundred and ninety adult patients were included in this analysis conducted retrospectively, based on available data, and collected prospectively. Clinical characteristics of the patients are presented in Table 1. Patients were treated according to the criteria defined by the European Bone Marrow Transplantation/European Conference on Infections in Leukemia. Briefly, they received fludarabine, busulfan, ATG for reduced-intensity conditioning (RIC) regimen, as previously described (9), or cyclophosphamide plus total body irradiation (TBI) for myeloablative conditioning regimen. Patients received cyclosporine A, mycophenolate mofetil, and/or methotrexate for usual prophylaxis of acute GVHD. Data regarding demographics, underlying disease, date and type of transplant, regimen, use of ATG or fludarabine, median number of CD34 cells ($\times 10^6/\text{kg}$) infused, date of EBV-R and other viral infections, rituximab infusions, lymphocyte phenotype were collected and available in our institutional database for each patient.

Diagnosis of EBV-reactivation and its treatment

EBV viral load (EBV-VL) was monitored in PB, for all the patients after HSCT, weekly for the first 6 months, and then monthly using standard PCR technique (10). EBV-R was defined by an EBV-VL superior or equal to 500 copies/mL, increasing 1 wk later. This was used to decide therapeutic intervention, first gradually reducing the doses of immunosuppressive drugs (20–25% reduction every 5 d on 1 month), followed by stopping them according to GVHD status and rituximab infusion (375 mg/m², four injections weekly), in case of no EBV-VL decrease with possible re-infusion if no EBV-VL control (11). CMV co-infection was defined if it occurred 1 month before or after the

Table 1 Patients characteristics

	Whole population	Patients with EBV-R	Patients without EBV-R	<i>P</i>
<i>N</i>	190	86	104	
Sex				
Female	76	30	46	NS
Male	114	56	58	
Median age at ASCT (years) (IQR) [range]	51 (38–58) [18–69]	48.5 (37–57) [18–68]	51 (40–60) [18–69]	NS
Diseases				
Aplastic anemia	5	4	1	NS
CML	9	5	4	
Acute leukemias and MDS				
Total	105	47	58	
ALL	26	14	12	
AML	71	29	42	
MDS	8	4	4	
Lympho-proliferative disorders				
Total	71	30	41	NS
Lymphoma	17	10	7	
CLL	8	3	5	
Myeloma	46	17	29	
Type of conditioning regimen				
Myeloablative	52	24	28	NS
RIC	138	62	76	
Type of graft				
PBSC	141	64	77	0.06
UCB	33	11	22	
Bone marrow	16	11	5	
Donor				
Unrelative donor	77	39	38	NS
Relative donor	80	36	44	
Cord blood	33	11	22	
Use of ATG				
Yes	111	60	51	0.004
No	79	26	53	
Use of Fludarabine				
Yes	133	60	73	NS
No	57	26	31	
Median of CD34 cells ($\times 10^6/\text{kg}$) (IQR)	4.28 (3.5–5)	4.14 (3.6–5)	5 (3.5–5)	NS

IQR, interquartile range; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; MDS, Myelodysplastic syndromes; CLL, chronic lymphocytic leukemia; RIC, reduced-intensity conditioning regimen; PBMC, peripheral blood mononuclear cells; UCB, umbilical cord blood cells; NS, not significant; ASCT, hematopoietic stem cell transplantation.

EBV-R. Acyclovir was used during the period before any demonstration of rituximab efficacy.

Lymphocyte immunophenotyping

Peripheral blood lymphocyte subpopulations (CD3+, CD3+CD4+, CD3+CD8+, CD3+CD56+, CD3-CD56+,

CD19, CD25+, HLA-DR+) were measured weekly for 3 months and monthly afterward using standard flow cytometry. For patients with EBV-R, we have chosen lymphocyte counts at the closest time of the diagnosis of EBV-R. Because the median time of EBV-R diagnosis is 73 d (IQR: 40–210) after HSCT, we considered this time for lymphocyte counts in patients with no EBV-R. Thus, we compare the NK cell counts according to EBV status of the patients. For patients receiving rituximab, lymphocyte counts were measured before the infusion or at least 2 d after the therapy.

Statistical analysis

Overall survival was defined from date of HSCT to death. PFS was defined from date of HSCT to relapse or death. Patients were followed until October the 10th of 2011. Survival was estimated with the Kaplan–Meier method. We compared survival between groups with the log-rank test. Median follow-up is carried out using reverse Kaplan–Meier method. Comparisons between qualitative variables have been conducted using chi-square tests. To compare means between two groups, we used Wilcoxon nonparametric test (two groups to be compared) or Kruskal–Wallis test (more than two comparison groups).

To observe the evolution of the rate of lymphocytes over time, we used a mixed model for repeated measures, and the *P*-value of a posteriori tests was corrected with Tukey's method. In order to show interdependency between the parameters, a multivariate analysis was performed for OS and PFS including parameters such as EBV-R, ATG usage. All analysis was performed with SAS statistical software (version 9.3, SAS institute Inc, Cary, NC, USA).

Results

Diagnosis of EBV-reactivation and its treatment

Eighty-six of the 190 patients (45%) presented EBV-R, with 74 of these 86 patients (86%) having a VL higher than 1000 copies/mL. There was no statistical difference between the two groups of patients, that is, having or not EBV-R, for age, sex, cancer type, conditioning regimen, in particular the use of fludarabine, type of HSCT, and number of infused CD34+ hematopoietic progenitors. Patients with EBV-R received more frequently ATG ($P = 0.004$) (Table 1). The median time of EBV-R detection was 73 d (IQR: 40–210) after HSCT. In case of EBV-R, the dose of immunosuppressive drugs was reduced before stopping them. Sixty-nine patients of the 86 patients received rituximab, and the other patients ($n = 17$) received acyclovir. Patients with no EBV-R did not receive rituximab therapy. Within the 3 months after rituximab therapy, GVHD intensity was decreased, particularly for acute GVHD, which was less frequent after rituximab therapy: 29 patients (42%) who presented grade 1

or 2 acute GVH, two patients (3%) having grade 3–4 after rituximab therapy compared with 58 patients (84%) with grade 1–2 acute GVHD, and 11 patients (16%) with grade 3–4, before rituximab therapy. In addition, at the time of EBV-R, all patients with EBV-R had GVHD symptoms, and only 38 patients (55%) had GVHD after rituximab therapy. The number of patients who underwent a CMV infection was not statistically different between the two groups of patients, that is, with EBV-R and with no EBV-R (respectively 8/86 vs. 20/104, $P = 0.49$).

Overall survival and progression-free survival

The median follow-up was 36.6 months (95% IC 31.5–45.7). We analyze different factors in both univariate and multivariate analysis, that is, clinical characteristics, use of ATG, use of rituximab, EBV status, type of graft and conditioning regimen, type of disease, circulating lymphocyte subpopulations. The use of ATG was not a significant factor on OS in univariate ($P = 0.93$) and multivariate analysis ($P = 0.81$). So the risk of death for patients with no EBV-R was superior than that observed for patients with EBV-R, after adjusting on NK and ATG administration (HR = 1.56, CI 95% = [0.944; 2.578]. Similarly, the use of ATG was not a significant factor on PFS in univariate ($P = 0.47$) or multivariate analysis ($P = 0.68$). The risk of relapse for patients with no EBV-R is superior than that observed for patients with EBV-R, when adjusting on NK and ATG (HR = 1.456, CI 95% = [0.952; 2.226]. No significant differences in PFS (Fig. 1A) and OS (Fig. 1B) were observed between patients having or not having rituximab (respectively $P = 0.867$ and $P = 0.669$).

Patients with EBV-R had a longer PFS than those with no EBV-R (at 2 yr 51% vs. 69%, at 5 yr 47% vs. 38%) ($P < 0.04$) (Fig. 2A). Similarly, OS was longer in patients with EBV-R than that observed for patients with no EBV-R (at 2 yr 76% vs. 64% and at 5 yr 63% vs. 47%, $P < 0.001$) (Fig. 2B). Accordingly to the OS difference, a lower number of deaths were observed in the group of patients having EBV-R than in the group without EBV-R, respectively 27/86 (31%) vs. 41/104 (39%). This tendency was probably linked to the effect of rituximab on reducing the intensity of GVHD with no significant advantage. As shown in Table 2, only sex and EBV status had a trend for statistical significance. So, males had 1.6 [0.969; 2.711]-fold higher risk to die than that observed for females. Patients with EBV-R had 1.6 [0.965; 2.726]-fold higher risk to die than EBV positive patients. If we introduce the sex in the model, EBV effect became statistically significant. Independently to the sex, EBV positive patients had 1.7 [1.045; 2.791]-fold higher risk to die than EBV negative patients. There were no differences for causes of deaths between the two groups of patients, that is, respectively, 10/27 vs. 14/41 deaths due to relapse, and only a tendency for higher risk of treatment-related mortality

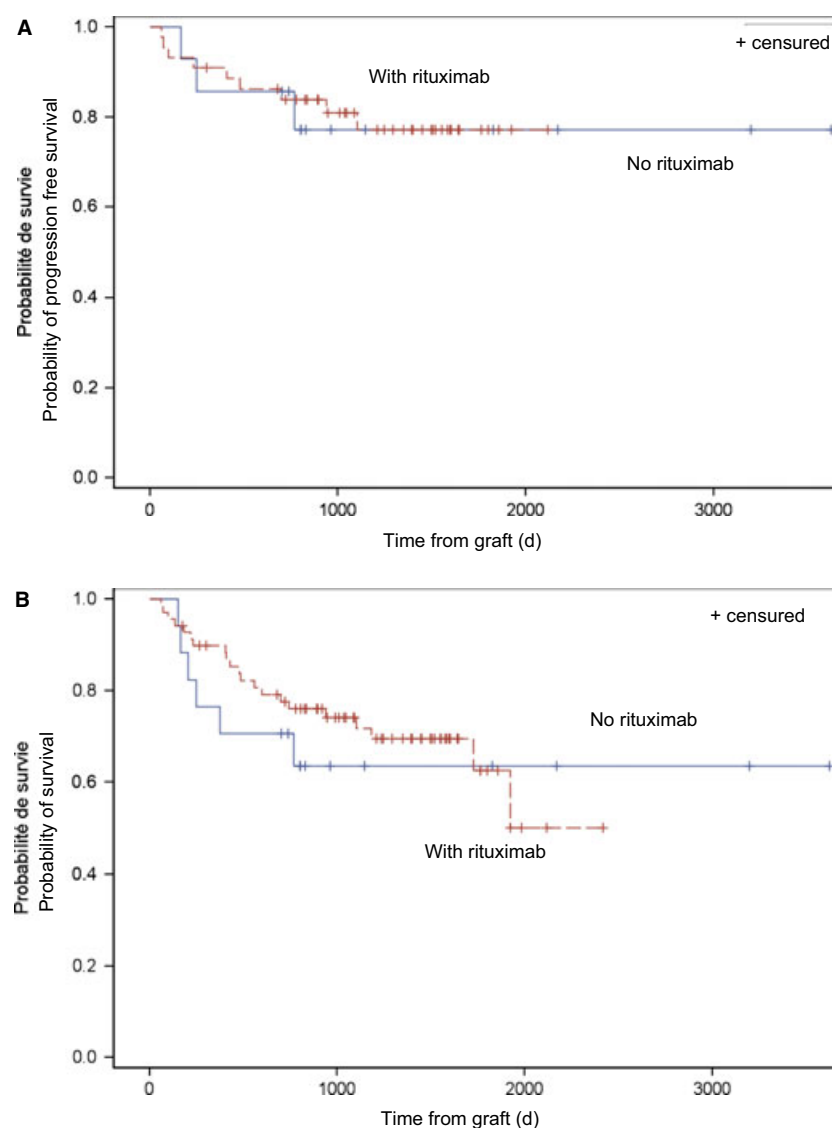


Figure 1 Progression-free survival (A) and overall survival (B) according to rituximab administration.

(TRM) for patients with EBV-R than that observed for patients without EBV-R (respectively 16/27 and 26/41, $P = 0.0763$, log-rank test). This tendency was probably linked to the effect of rituximab on reducing the intensity of GVHD with no significant advantage. So, among the patients with EBV-R, OS and PFS were not significantly different for patients receiving or not rituximab (respectively $P = 0.67$ and $P = 0.88$). OS and PFS were not different between patients according to the type of hematological malignancy (respectively, $P = 0.46$ and $P = 0.49$).

Lymphocyte immunophenotyping

The median duration of lymphocyte measurement was 94 d (IQR 59–248) after HSCT. B lymphocytes and particularly T lymphocytes, including the different T-cell subpopulations, that is, CD4+ and CD8+ lymphocytes, were not significantly different between the two groups of patients (respectively

$P = 0.29$ and $P = 0.48$). Only circulating NK cells were significantly different, with a median increased by 22% ($P = 0.03$) in patients with EBV-R, 150 cells/mL (IQR: 80–255) as compared to 123 cells/mL (IQR: 68–182) in patients with no EBV-R (Fig. 3). CMV co-infection did not impact NK cell counts [$P = 0.58$; $N = 150$ vs. $N = 40$ median 137.9 (75.46; 215.02) vs. 123.3 (72.85; 228.34)]. NK cell counts were not different according to the use of rituximab ($P = 0.22$). The use of rituximab did not modify NK cell counts at 3 and 6 months after stopping treatment (both $P = 0.99$). There were no significant differences for circulating NK cells between the different groups of patients (EBV-R and no EBV-R) when stratified on ATG administration ($P = 0.1468$).

Discussion

Hematopoietic stem cell transplantation is a complex therapy that associates immune modulation to obtain a control of the

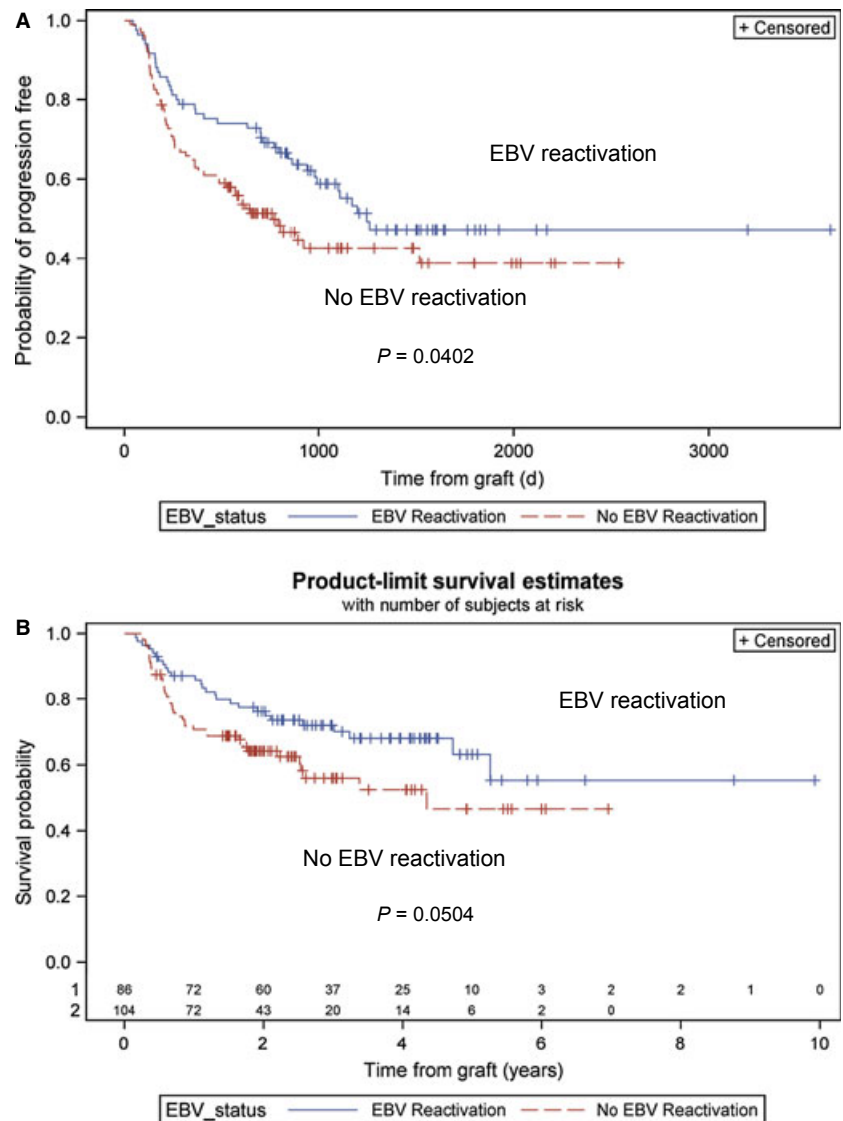


Figure 2 Progression-free survival (PFS) (A) and overall survival (OS) according to EBV-reactivation (B).

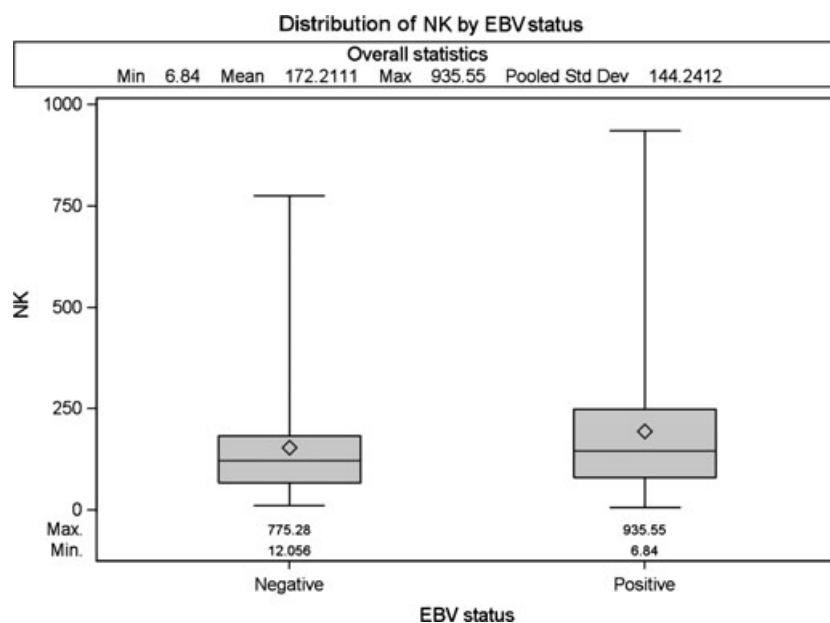
residual disease. So, patients balance between two clinical risks, relapse, and GVHD, generally associated with infections. Among them, CMV infection or reactivation and/or EBV-R constitute major risks for these patients. Most of our patients received rituximab infusion as a preemptive therapy, and the other patients received acyclovir during the pre-rituximab period, thus representing a control group. Several risk factors have been described to favor EBV-R and EBV-PLTD, but ATG is the major one (11, 12). Peric *et al.*, also found that ATG is the main risk factor for EBV-R, and rituximab overcomes its risks. However, they showed no difference in survival for patients with EBV-R as compared to patients without EBV-R, but criteria for defining EBV-R were different and the number of patients having EBV-R was too short as well as the duration of follow-up (13). If rituximab may control EBV-VL and GVHD, we did not demonstrate any impact of rituximab infusion on PFS and

OS. In addition, patients with EBV-R who received acyclovir had similar PFS and OS compared with those who received rituximab. However, rituximab reduced the intensity of GVHD, despite the fact that TRM was not significantly different between the two groups of patients, that is, with or without EBV-R. So, rituximab may have an additional effect to other factors on PFS and OS. The number of circulating NK cells was significantly increased in patients with EBV-R, whether or not they received rituximab. NK cells are major cells implicated in antibody-dependent cell-mediated cytotoxicity (ADCC) through their CD16 expression. Activated circulating NK cells were shown to increase within 4 h of rituximab infusion, as measured by CD54 expression (14), particularly for subjects with the high-affinity CD16 polymorphism (15). In our study, NK cells measured in PB represented the whole population, independently to the expression of any marker of activation. This observation

Table 2 Multivariate analysis

Variable	Class	Hazard ratio	IC 95%	P-value of the comparison	Overall P-value
ATG	Negative	1			0.9453
	Positive	0.971	[0.419; 2.251]	0.9453	
Type of graft	Standard conditioning regimen	0.943	[0.428; 2.078]	0.8835	0.6888
	RIC (mini-AT)				
	RIC (micro-AT)	0.671	[0.232; 1.943]	0.4617	
	Sequential transplantation (Autologous followed by HSCT)	0.378	[0.051; 2.803]	0.3411	
NK (/mm ³)	<135	1			0.3622
	≥135	0.789	[0.474; 1.314]	0.3622	
Type of graft	Bone marrow	1			0.3509
	PBSC	2.441	[0.723; 8.24]	0.1506	
	UCB	2.119	[0.438; 10.262]	0.3508	
EBV	Positive	1			0.068
	Negative	1.622	[0.965; 2.726]	0.068	
Sex	Female	1			0.0655
	Male	1.621	[0.969; 2.711]	0.0655	

ATG, antithymocyte globulin; RIC, reduced-intensity conditioning regimen; PBMC, peripheral blood mononuclear cells; UCB, umbilical cord blood cells; HSCT, hematopoietic stem cell transplantation.

**Figure 3** Box plot of NK cells count in patients with EBV-reactivation (EBV-R) vs. patients with no EBV-R.

may support the fact that EBV probably amplifies NK cell compartment and activates these cells to get an antitumoral effect. This hypothesis has been suggested for CMV in an animal model (16) and is now tested in our group. It is well known that the adaptive way of the immune system plays a pivotal role in protective immunity against EBV (17, 18). NK cells are particularly active for controlling EBV-transformed cells. Early after primary viral infections, NK cells limit the viral burden until virus-specific T cells are able to eliminate the infection or control viral titers at low levels, particularly with cytokine and chemokine production (e.g., IFN- γ , TNF- α , MIP-1 α , MIP-1 β , and RANTES), and to

interact with dendritic cells (19). NK cells are the first lymphoid cells to repopulate, reaching normal numbers within 1 month regardless of donor type or patient age in adults and children (20). Two major functional NK cell subsets have been observed in humans: CD16⁺CD56^{dim} NK cells (90% of the circulating NK cells), which lyse susceptible target cells, but secrete low levels of cytokines after activation, and CD16⁻CD56^{bright} NK cells (<10% of the circulating NK, but dominant in secondary lymphoid organs) that produce large amounts of cytokines upon stimulation and acquire cytotoxicity only after prolonged activation (21, 22). Till now, only NK cell allo-reactivity, defined by the

absence of recipient MHC class I ligands for donor inhibitory killer immunoglobulin-like receptor (KIR), has been shown to activate these cells in order to participate to tumor control, as well as to an antiviral effect (23–25). Most donors have the potential to exert NK alloreactions as they possess a full complement of inhibitory KIR genes.

In the context of HSCT, this is the first study that demonstrates a survival benefit due to controlled EBV-R, probably linked to an amplification and/or activation of NK cells. In EBV and CMV infection, the virus encodes a protein that mimic class I HLAs and that interfere with the expression of ligands for different activating receptors (e.g., NKG2D, DNAM-1, and natural cytotoxicity receptors such as Nkp46 (26, 27). Recently, it has been shown that CMV reactivation after ASCT promotes a lasting increase in educated NKG2C positive NK cells (16). In addition, it has been demonstrated in a mouse model that activated NK lymphocytes with NKp30-, NKp46-, NKG2D-, and DNAM-1-activating receptors expression control myeloma cells (28). Viruses including EBV and CMV represent great stimulators for both function and amplification of NK compartment (29). The advantage of EBV is that the reactivation of such a virus is early detected and potentially controlled by rituximab administration, which is not the case with other viral reactivation or infection such as CMV, another frequent virus in the setting of HSCT (30). So, a controlled EBV-R is beneficial for the patients who need an additional antitumor control, and NK cells may exert such an activity. This opens the way for using NK cell drugs, as it is now the case in different early clinical trials (31) or to use EBV-derived molecules for *in vivo* amplifying the number and the activity of NK cells.

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Conflict of interest

No conflict of interest.

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